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WO 01/83551 A1

(54) Title: REAGENT FOR DETECTION OF BIOMOLECULES, AND USE THEREOF

(57) Abstract: A cell-free reagent for detection of a target molecule is disclosed. Said reagent comprises a binding domain linked to a reporter domain. The binding domain has affinity to the target molecule and will bind to it when brought in contact therewith. The binding of the binding domain to the target molecule will induce dimerization of one reagent molecule with another reagent molecule with a similar binding domain, and as a result the reporter domain will induce a measurable signal. The binding domain may e.g. be the extracellular domain of a receptor, and the reporter molecule may be the intracellular domain of another receptor. Also the use of said receptor for the determination of the presence and/or the level of a target molecule present in a fluid sample and for the screening of potentially pharmaceutical active biomolecules is disclosed.

REAGENT FOR DETECTION OF BIOMOLECULES, AND USE THEREOF

Field of the invention

The present invention relates to a new cell-free reagent for detection of biomolecules, and in particular of receptor ligands. The invention also relates to use of said reagent.

Description of background and related art

The activation of several receptors is initiated by dimerization of two receptor molecules. Examples of receptors where activation is dependent receptor dimerization include receptors of the cytokine receptor superfamily and the tyrosine kinase superfamily. Receptors of the receptor tyrosine kinase family are single-pass transmembrane proteins with intrinsic enzymatic activity. The receptors consist of an extracellular domain which binds ligands, a transmembrane domain and a cytoplasmatic or intracellular domain which has enzymatic activity. The cytoplasmatic domain of the tyrosine kinase receptors can catalyze the transfer of phosphate from ATP to tyrosine residues in protein substrates. Activation of the receptors is initiated by ligand binding, which facilitates receptor dimerization and results in phosphorylation of tyrosine residues in the intracellular domain of the receptor protein. The phosphorylated tyrosine residues enhance the catalytic activity of the receptor or serve as docking sites for downstream signaling molecules. The tyrosine kinase receptor superfamily includes several growth factor receptors, e.g. receptors for epidermal growth factor (EFG), fibroblast growth factor, platelet derived growth factor, and vascular endothelial growth factor.

Receptors of the cytokine superfamily are single chain transmembrane receptors consisting of an extracellular ligand-binding domain, a transmembrane domain and

an intracellular cytoplasmatic domain. The GH receptor (GH-R) is a member of the cytokine receptor super-family, which also includes, amongst others, the receptors for prolactin, leptin, erythropoietin, macrophage colony-stimulating factor, interleukins and leukemia inhibitor factor. In contrast to the receptors of the tyrosine kinase superfamily, the cytoplasmatic domain of the cytokine receptors lacks intrinsic enzymatic activity.

In clinical medicine and for research purposes, the concentration of ligands for various receptors is often measured in biological fluids. So far, this has mainly been done using immunoassay based on antibodies capable of binding to the ligand. However, such assays measure both biologically active and inactive molecules.

WO 94/29458 describes hybrid receptor molecules wherein one domain of the receptor is derived from the cytokine superfamily of receptors and another domain is derived from an unrelated family of receptors. One of the domains is a receptor extracellular domain and the other is a receptor intracellular domain. These hybrid receptor molecules also comprises an transmembrane domain derived from the same receptor as either the intracellular or extracellular domain, or it may be derived from a third receptor source. The hybrid receptor molecules according to WO 94/29458 are then transfected into host cell lines before they can be used for, for example, screening for receptor ligands. A major disadvantage with these hybrid receptor molecules is that they are transfected into cells and must be used as a cell culture, and thus cannot be used in solution.

Summary of the invention

The object of the present invention is to provide a reagent based on type of hybrid receptor that can be used in solution.

The method according to the present invention is based on the use of a dimerizable, or optionally oli-

gomerizable, molecule, preferably a biomolecule, comprising or consisting of a soluble chimeric receptor or part of such a receptor linked to a molecule which has an activity or other feature that is changed after dimerization of two such biomolecules. The reagent according to the present invention has the advantage, compared to hitherto utilized reagents such as antibodies, that it recognizes molecules that activate or inhibit receptors.

Another advantage of the invention is that it is possible to use the chimeric receptor in solution instead of on cells or transfected into cells to obtain a signal caused by a ligand for the receptor.

The inventors have also for the first time shown that is that it is not necessary to include the transmembrane domain of a membrane-bound receptor in a reagent.

The reagent according to the invention can be used to measure the concentration of biochemically active ligand.

It can also be used to search for unknown molecules which can bind to and activate or inhibit the receptor of interest. In the search for such ligands, the reagent according to the present invention may replace the cell based high throughput screening systems commonly used in pharmaceutical research to identify candidates for drug development.

In the research work leading to the present invention the inventors have for the first time recognized that many of the problems associated with the use of chimeric receptors expressed on cells can be overcome by using such receptors as reagent in a cell free system.

Thus, the present invention relates to a cell-free reagent for the detection of target molecules, said reagent comprising a binding domain linked to a reporter domain, wherein said binding domain has affinity to said target molecule and will bind to said target molecule when brought in contact therewith, and wherein the binding of said binding domain to said target molecule will

induce dimerization or oligomerization of the reagent molecule with another reagent molecule with a similar binding domain, and wherein said reporter domain will induce a measurable signal upon said dimerization or oligomerization

The invention also relates to a method for determination of the presence and/or the concentration of a specific target molecule in a sample, such as a body fluid, or more precisely to the use of the above mentioned reagent for the determination of the presence and/or the concentration of a specific target molecule in a sample.

The invention also relates to a method for screening of potentially pharmaceutical active biomolecules, or more precisely to the use of the above mentioned reagent for the screening of potentially pharmaceutical active biomolecules.

The characterizing features of the invention will be evident from the following description and the appended claims.

Detailed description of the invention

As stated above the invention relates to reagent for detection of a target molecule, said reagent comprising a binding domain linked to a reporter domain, wherein said binding domain has affinity to said target molecule and will bind to said target molecule when brought in contact therewith, and wherein the binding of said binding domain to said target molecule will induce dimerization or oligomerization of the reagent molecule with another reagent molecule with binding domain, and wherein said reporter domain will induce a measurable signal upon said dimerization or oligomerization.

The binding domain of the reagent according to the invention may be any kind of molecule or structure that has affinity for the target molecule to be detected. It may, for example, be any kind of receptor or part of receptor. When the binding domain is constituted of a part

of a receptor, this part should comprise the specific binding site of the receptor in question. Non-limiting examples of such a receptor suitable for forming the binding domain of the reagent according to the invention are growth hormone receptors (GH-R), erythropoietin receptors and EGF receptors (EGF-R).

The reporter domain of the reagent according to the invention may be any kind of molecule or structure that in some way will lead to a measurable signal upon dimerization, or oligomerization, of the reagent. The reporter domain should thus have an intrinsic activity or other feature which is possible to detect after dimerization. It may also have a measurable activity prior to dimerization, and in this case the activity will be altered by the dimerization. Preferably the target molecule is a biomolecule, which must be capable of dimerizing or oligomerizing the reagent. Non-limiting examples of such ligands are hormones, receptor agonists, receptor antagonists, antigens, antibodies, and non-peptide molecules.

The binding domain and the reporter domain of the reagent according to the invention may be linked directly to each other. They may also be linked to each other via any kind of spacer, provided that this spacer does not have any negative influence on the affinity of the binding domain to the target molecule or on the dimerization or oligomerization of the reporter domain or on the inducement of the measurable signal.

The measurable signal induces upon dimerization or oligomerization may e.g. be the activation of an enzyme, which modifies a substrate. The modified subtrate may be detected by several methods known to man skilled in the art, e.g. spectrophotometrically or as incorporated radioactivity.

The sample analyzed with the reagent according to the invention may be any fluid sample, such as a body fluid or a solution of different biomolecules.

In preferred embodiment of the invention the reagent consists of a fusion protein formed between the extracellular domain of the human growth hormone receptor linked to the intracellular domain of the human epidermal growth factor receptor, for which the intrinsic enzymatic activity is activated when two such molecules are dimerized by a ligand.

Another example of specific reagent according to the invention is a fusion protein between the extracellular domain of a cytokine receptor linked to the intracellular domain of a tyrosine kinase receptor, for which the intrinsic enzymatic activity is activated when two such molecules are dimerized by a ligand.

The reagent may be used in the form of a solution. However, in some cases it may be advantageous to attach the reactant to a solid carrier, such as a plate or test tube.

The reagent according to the invention is suitable for detection and concentration determination of a specific target molecule in a sample.

The reagent according to the invention may also be used in assays for measuring the concentration of the target molecule, wherein the reagent replaces the traditionally used antibodies. For this purpose, the reagent is exposed to unknown amounts of the target molecule in a sample and the signal produced is compared to the signal in a standard curve constructed by activation of the fusion protein by known amounts of a target molecule.

Examples

The invention is further illustrated in the examples below, which in no way are intended to limit the scope of the invention.

In the examples reference is made to the appended drawings on which:

Fig. 1 illustrates Xba-Kpn I and Hind III-Kpn I digestion of pAL-1-48. Clone number identifies the

specific colony from which the plasmid was purified and "uncut" refers to undigested plasmid;

Fig. 2 illustrates Xba I-Hind III digestion of pAL-1-48;

Fig. 3 illustrates detection of GH using cell lysates containing a recombinant reagent consisting of a GH-R extracellular domain/EGF-R intracellular domain fusion protein; and

Fig. 4 illustrates detection of GH using purified recombinant reagent consisting of a GH-R extracellular domain/EGF-R intracellular domain fusion protein.

In the examples, the following abbreviations, which are well known to man in the art, are used:

GH-R = growth hormone receptor
hGH-R = human growth hormone receptor
EGF-R = epidermal growth factor receptor
hEGF-R = human epidermal growth factor receptor
PCR = polymerase chain reaction

Example I

In this example, a reagent consisting of a GH-R/EGF-R fusion protein was produced.

To produce the reagent, a DNA construct was made with a cDNA sequence corresponding to the extracellular domain of the human GH-R (hGH-R) ligated to a cDNA sequence corresponding to the intracellular domain of the human epidermal growth factor receptor (hEGF-R). The recombinant GH-R/EGF-R chimeric receptor was expressed in a baculovirus system using methods known to man skilled in the art.

Generation of the *baculovirus expression vector for the GH-R/EGF-R fusion protein*

cDNA encoding the extracellular domain of the hGH-R was obtained by PCR using primers 5'-GGTCTAGAACCATGGGACATCACCATCACCATCACTTTTCTGGAAGTGAGGCCA-3' and 5'-AGGTACCTTGGCTCATCTGAGGAAGT-3' (the underlined sequences contain restriction enzyme sites for Xba I and

Kpn I, respectively and the bold sequence encodes a repeat of six histidine residues), based on the nucleotide sequence of the hGH-R, using a plasmid, containing the hGH-R cDNA modified by site-directed mutagenesis to allow efficient replication in E. coli, as template. Amplification was done in Taq extender buffer (10 mM KCl, 20 mM Tris-HCl (pH 8,8), 10 mM NH_4SO_4, 2 mM $MgSO_4$, 0,1% triton X-100, 0.1 mg/ml BSA), a mixture of Taq polymerase (2.5 U) (Boehringer Mannheim, Mannheim, Germany) and Pfu (1.25 U) (Stratagene, La Jolla, CA, U.S.A.), primers (50 µM each), human liver cDNA, dNTP (0.2 mM each), using a PCR GeneAmp System 9600 (Perkin Elmer, Foster City, CA, U.S.A.), programmed for 20 cycles with a denaturation step at 94°C for 20 s, annealing at 55°C for 20 s and an extension step at 72°C for 1 min and 30 s.

The hEGF-R was cloned by PCR using primers 5'-AGGTACCCGAAGGCGCCACATC GTT-3' and 5'-TCTCAAGCTTTCAGTGATGGTGATGGTGATGTGCTCCAATAA ATTCACT-3', (the underlined sequences contain restriction enzyme sites for Kpn I and Hind III, respectively and the bold sequence encodes a repeat of six histidine residues), based on the nucleotide sequence of the hEGF-R, using cDNA as template. Amplification was done as described above. The PCR GeneAmp System was this time programmed for 20 cycles with a denaturation step at 94°C for 20 s, annealing at 55°C for 20 s and an extension step at 72°C for 3 min. The primers used were purchased from Scandinavian Gene Synthesis AB, Köping, Sweden.

The hGH-R and EGF-R DNA fragments were cloned into pCRII (Invitrogen, San Diego, CA, U.S.A.), yielding pAL-1-26 and pAL-1-32, respectively. The E. coli strain DH5-alpha was used for amplification of plasmid DNA, using the expression of plasmidic lacZ indicator gene for selection of colonies containing the insert. Transformation and plasmid purification (QIAprep Spin Miniprep Kit, Qiagen, Germany) were performed. Inserts of correct size were verified by restriction enzyme digestion. Eco RI di-

gestion of pAL-1-26 generated a fragment corresponding to the expected size of 800 bp, whereas Hind III-Eco RV digestion of pAL-1-32 revealed a fragment of 1600 bp, as expected.

Construction of expression-vectors

The plasmid pAL-1-26 was digested with Kpn I and Xba I and the released fragment was gel-purified and subcloned into a Kpn I- Xba I digested pFAST (Gibco, Paisley, UK.), yielding the plasmid pAL-1-38. As before, E. coli strain DH5-alpha was used for amplification of plasmid DNA. To identify clones with a correct insert, restriction enzyme digestion was performed. Hind III-Bam HI digestion revealed an insert of expected size and Hind III-Eco RV digestion verified correct orientation of the fragment.

The pAL-1-32 plasmid was digested with Kpn I- Hind III, the released fragment was gel purified and subcloned into a Kpn I- Hind III digested pAL-1-38 yielding the plasmid pAL-1-48. To verify clones with correct insert, digestion was done with Xba I-Kpn I (releasing the GH-R fragment), Kpn I-Hind III (releasing the EGF-R fragment) (Fig. 1) and Xba I-Hind III that released the fusion protein DNA construct (Fig. 2). Clone 9 was selected for further work.

Verification by sequencing

Sequencing of the plasmids pAL-1-26, pAL-1-32, pAL-1-38 and pAL-1-48 was performed using of the PRISM BigDye Terminator Sequencing Kit (Applied Biosystem Division, Perkin Elmer, Foster City, CA, U.S.A.) and an ABI 377A automatic sequencer (Applied Biosystem). The DNA sequences were aligned to the corresponding DNA sequences obtained from Genebank. No mismatches were found.

Protein expression

Recombinant protein was expressed using baculovirus, Bac-to-Bac expression system (GIBCO, Paisley, U.K.) using methods known to man skilled in the art. All reagents for cell culture and transfection were from GIBCO.

Example II

In this example, a cell free bioassay for human GH was developed using a reagent according to the invention.

In contrast to the cytokine receptors (e.g. GH-R), receptors of the tyrosine kinase family (e.g. EGF-R) possess intrinsic tyrosine kinase activity and are therefore not dependent on other proteins for enzyme activity. Therefore, a GH-R extracellular domain/EGF-R intracellular domain fusion protein was used as the reagent to detect GH in a sample.

Lysates from cells expressing the recombinant GH-R/EGF-R fusion protein was added to samples containing recombinant human GH and to control samples containing no GH. A solution containing a biotinylated peptide substrate and radiolabeled ATP was added to the samples. After incubation at 30°C for 15 min, the reaction was stopped by adding termination buffer. The samples were pipetted on to streptavidin coated membranes. The membranes were washed and the radioactivity was counted. As shown in Fig. 3, the presence of GH in the samples resulted in increased phosphorylation of the substrate.

In a similar experiment, the recombinant GH-R extracellular domain/EGF-R intracellular domain fusion protein was purified from lysates from cells expressing the fusion protein using Ni-NTA columns (Qiagen, Germany) and the repeats of histidine residues (see Example I) as affinity tags.

Purified recombinant GH-R/EGF-R fusion protein was added to samples containing recombinant human GH and to control samples containing no GH. A solution containing a biotinylated peptide substrate and radiolabeled ATP was

added to the samples. After incubation at 30°C for 15 min, the reaction was stopped by adding termination buffer. The samples were pipetted on to streptavidin coated membranes. The membranes were washed and the radioactivity was counted. As shown in Fig. 4, the presence of GH in the samples resulted in increased phosphorylation of the substrate.

CLAIMS

1. A cell-free reagent for detection of a target molecule comprising a binding domain linked to a reporter domain, wherein said binding domain has affinity to said target molecule and will bind to said target molecule when brought in contact therewith, and wherein the binding of said binding domain to said target molecule will induce dimerization or oligomerization of the reagent molecule with another reagent molecule with a similar binding domain, and wherein said reporter domain will induce a measurable signal upon said dimerization or oligomerization.

2. A reagent according to claim 1, wherein said target molecule is a biomolecule.

3. A reagent according to claim 1 or 2, wherein said measurable signal is generated upon dimerization or oligomerization.

4. A reagent according to claim 3, wherein said measurable signal is caused by the activation of an enzyme upon said dimerization or oligomerization.

5. A reagent according to any one of the claims 1 - 4, wherein said binding domain is directly linked to said reporter domain.

6. A reagent according to any one of the claims 1 - 5, consisting of only said binding domain and said reporter domain.

7. A reagent according to any one of the claims 1 - 4, wherein said binding domain is linked to said reporter domain by means of a spacer.

8. A reagent according to any one of the claims 1 - 7, for the determination of the amount of biochemically active target molecule in a sample.

9. A reagent according to claim 8, wherein said sample is a body fluid.

10. A reagent according to any one of the claims 1 - 9, wherein said binding domain is constituted by at least a part of a receptor.

11. A reagent according to claim 10, for the identification of target molecules binding to the receptor.

12. A reagent according to any one of the claims 1 - 11, wherein said reporter domain is constituted by at least a part of a receptor.

13. A reagent according to any one of the claims 1 - 12, wherein said binding domain is the extracellular domain of a growth hormone receptor.

14. A reagent according to claim 13, wherein said binding domain is the extracellular domain of the human growth hormone receptor.

15. A reagent according to claim 13 or 14, for the detection of growth hormone.

16. A reagent according to any one of the claims 1 - 12, wherein said binding domain is the extracellular domain of an erythropoietin receptor.

17. A reagent according to claim 16, wherein said binding domain is the extracellular domain of the human erythropoietin receptor.

18. A reagent according to claim 16 or 17, for the detection of erythropoietin.

19. A reagent according to any one of the claims 1 - 12, wherein said binding domain is the extracellular domain of an epidermal growth factor receptor.

20. A reagent according to claim 19, wherein said binding domain is the extracellular domain of the human epidermal growth factor receptor.

21. A reagent according to claim 19 or 20, for the detection of epidermal growth factor.

22. A reagent according to any one of the claims 1 - 21, wherein said reporter domain is the intracellular domain of an epidermal growth factor receptor.

23. A reagent according to claim 22, wherein said reporter domain is the intracellular domain of the human epidermal growth factor receptor.

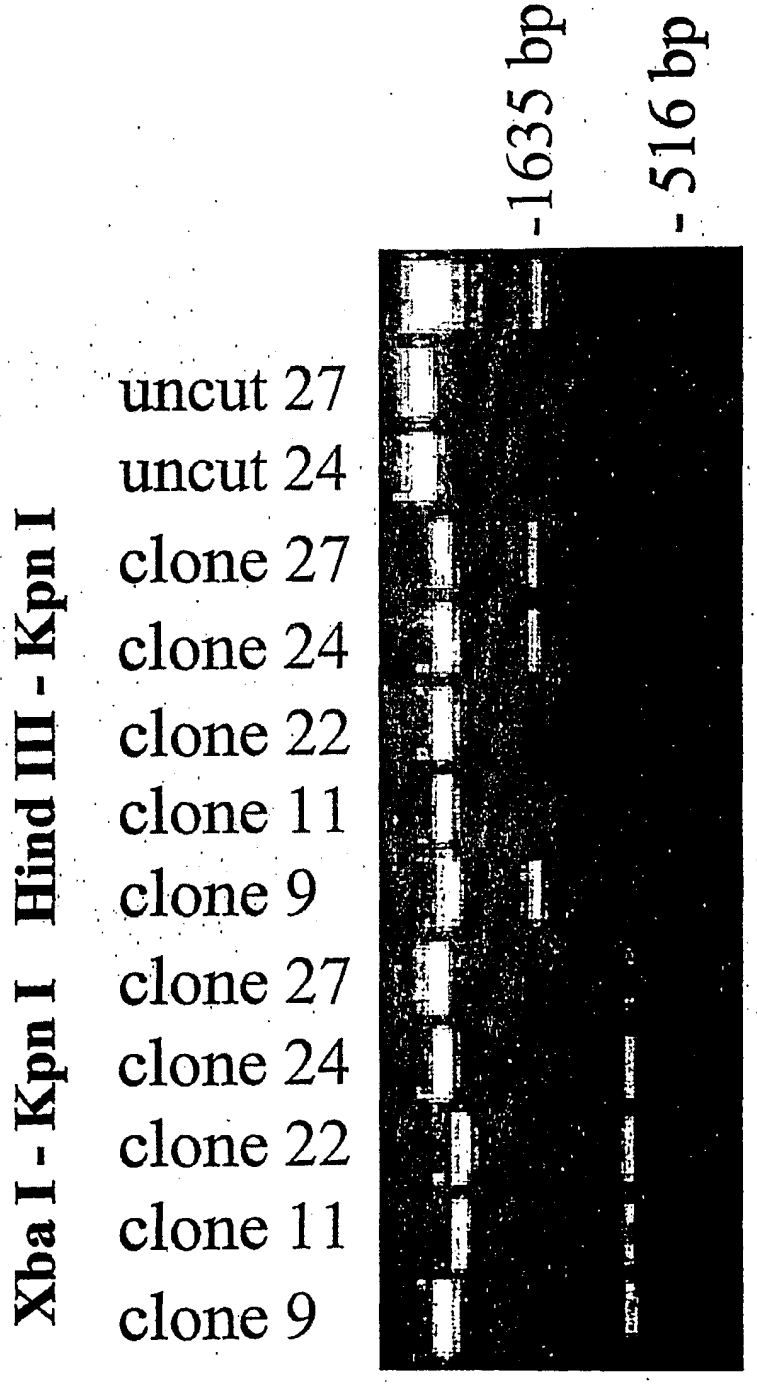
24. A reagent according to any one of the claims 1 - 21, wherein said reporter domain is the intracellular domain of a tyrosine kinase receptor.

25. A reagent according to claim 24, wherein said reporter domain is the intracellular domain of a human tyrosine kinase receptor.

26. Use of a reagent according to any one of the claims 1 - 25, for the determination of the presence and/or the level of a target molecule present in a fluid sample.

27. Use of a reagent according to any one of the claims 1 - 25, for the screening of potentially pharmaceutical active molecules.

28. Use according to claim 27 in the production of a pharmaceutical composition.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Xba I - Kpn I Hind III - Kpn I

Fig 1

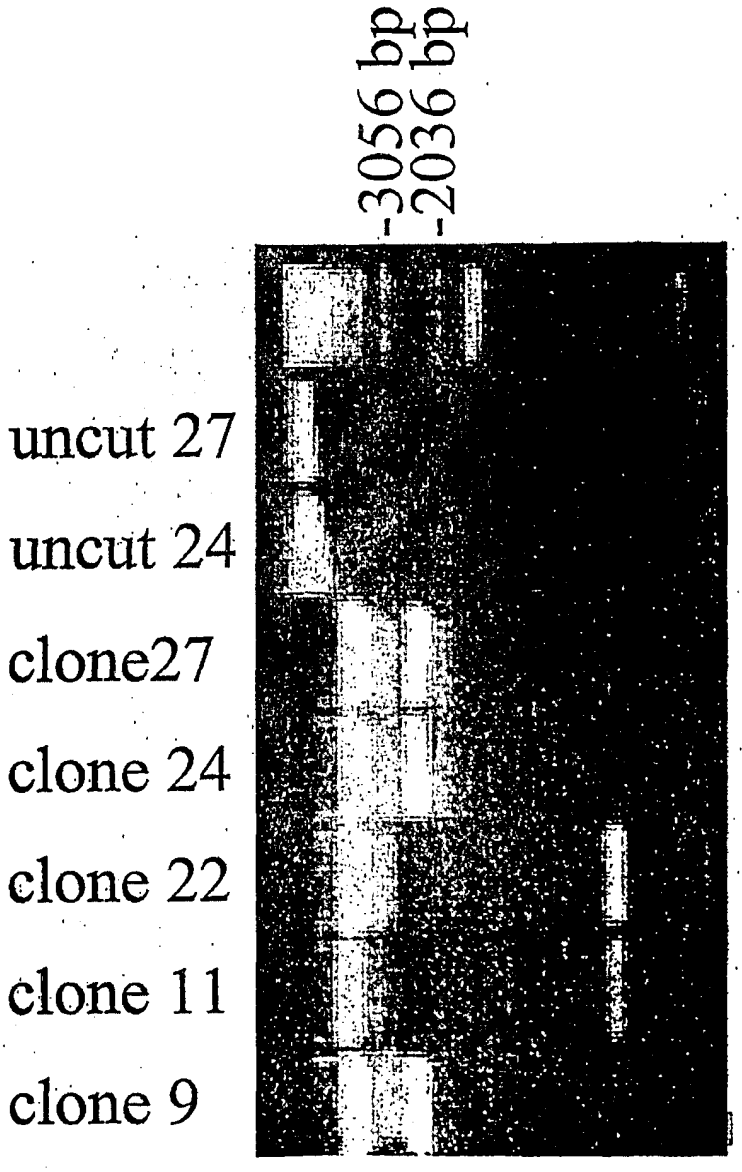
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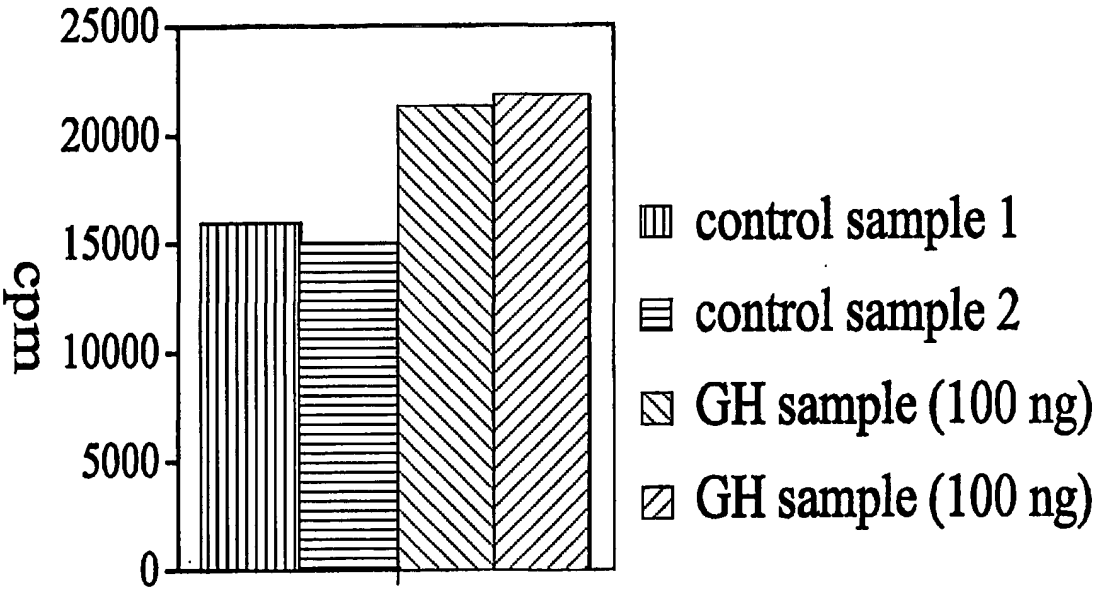
Fig 2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cpm
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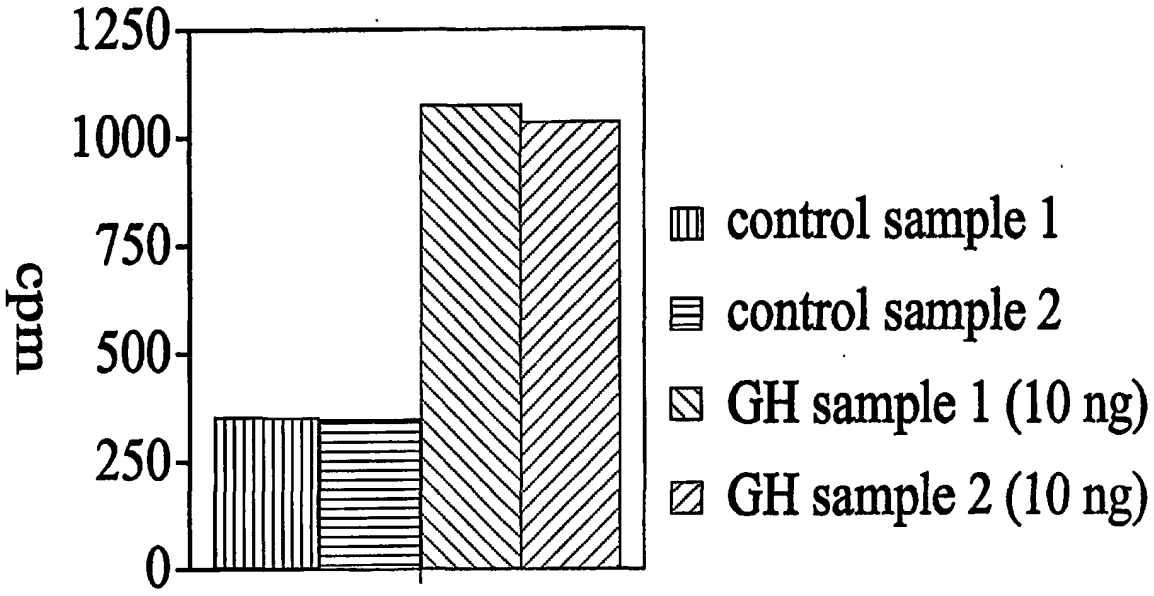
control sample 2
GH sample (100 ng)
GH sample (100 ng)

Fig. 3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750
500
250
0
cpm
control sample 1
control sample 2
GH sample 1 (10 ng)
GH sample 2 (10 ng)

Fig. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 01/00941

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 14/705, G01N 33/53, C07K 19/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K, G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9429458 A1 (AMGEN INC.), 22 December 1994 (22.12.94) --	1-28
A	EP 0950710 A2 (JCR PHARMACEUTICALS CO., LTD.), 20 October 1999 (20.10.99) --	1-28
A	WO 9740381 A1 (XENOGEN), 30 October 1997 (30.10.97) -- --------	1-28

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 Sept 2001	21 -09- 2001
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INTERNATIONAL SEARCH REPORT

Information on patent family members

03/09/01

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		AU 7203694 A	03/01/95
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EP 0950710 A2	20/10/99	JP 11285388 A	19/10/99
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		CN 1219239 A	09/06/99
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